Enhanced pre-operative axillary staging using intradermal microbubbles and contrast-enhanced ultrasound to detect and biopsy sentinel lymph nodes in breast cancer: A potential replacement for axillary surgery

Short title: Microbubble and CEUS core biopsy of sentinel nodes in breast cancer

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Abstract

Objectives

To compare the experience of 4 UK Centres in the use of intradermal microbubbles and contrast enhanced ultrasound (CEUS) to pre-operatively identify and biopsy sentinel lymph nodes (SLN) in patients with breast cancer.

Methods

In all centres, breast cancer patients had a microbubble/ CEUS SLN core biopsy prior to axillary surgery and patients in Centres 1 and 2 had a normal grey-scale axillary ultrasound. Data was collected between 2010 and 2016; 1361 from Centre 1 (prospective, sequential), 376 from Centre 2 (retrospective, sequential), 122 from Centre 3 (retrospective, selected) and 48 from Centre 4 (prospective, selected).

Results

SLN were successfully core biopsied in 80% (Centre 1), 79.6% (Centre 2), 77.5% (Centre 3) and 88% (Centre 4). The sensitivities to identify all SLN metastases were 46.9% (95% CI 39.4-55.1), 52.5% (95% CI 39.1-65.7), 46.4% (95% CI 27.5-66.1) and 45.5% (95% CI 16.7-76.6) respectively. Specificities; 99.7% (95% CI 98.9-100), 98.1% (95% CI 94.5-99.6), 100% (95% CI 93.2-100%) and 96.3%

(95% CI 81-99.9) respectively. Negative predictive values; 87.0% (95% CI 84.3-89.3), 84.5% (95% CI 78.4-89.5), 86.9% (95% CI 82.4-90.3) and 86.2% (95% CI 78.4-91.5) respectively. At Centres 1 and 2, 12/730 (1.6%) and 7/181 (4%) respectively of patients with a benign microbubble/ CEUS SLN core biopsy had 2 or more lymph node (LN) macrometastases found at the end of primary surgical treatment.

Conclusions

The identification and biopsy of SLN using CEUS is a reproducible technique.

Advances in knowledge

In the era of axillary conservation, microbubble/ CEUS SLN core biopsy has the potential to succeed surgical staging of the axilla.

Introduction

Sentinel lymph node excision (SLNE) is the axillary staging method of choice for breast cancer patients with normal axillary lymph nodes (LN) on grey-scale ultrasound or a benign biopsy of morphologically abnormal LN (1,2). Although SLNE has less reported morbidity than axillary lymph node dissection (ALND) it remains a surgical procedure performed under general anaesthesia with recognised immediate complications such as infection (11%) and long term problems with sensory loss (11%) and arm lymphoedema (5%) at 12 months (3) The operation is also reliant upon 2 tracers (radioactive isotope and blue dye) to locate SLN and maintain a false negative rate of 6% (4). The blue dye carries a hazard of anaphylaxis (0.9%) (5) and there are logistical challenges in obtaining medical grade radioisotopes.

Recently, the practice of removing all malignant axillary LN to achieve local control has been challenged by the results of a trial where patients with SLN metastases were randomized to a completion ALND or no further axillary surgery (6). The local recurrence rate in the axilla was low with no difference between the groups despite the fact that 27.3% of patients in the ALND arm had further lymph node metastases retrieved (6). These results emphasize the role of modern adjuvant treatment in preventing loco-regional disease recurrence and have led to the introduction of conservative surgical management of the axilla for patients with up to 2 SLN macrometastases (7).

In patients with breast cancer, SLN can be identified and percutaneously biopsied in the clinic using intradermally injected microbubbles and contrast-enhanced ultrasound (CEUS) (8, 9, 10). The technique was originally described in a swine melanoma model (11) and following a trial period, was incorporated

into routine practice at Centre 1 for all invasive breast cancer patients with a normal grey-scale axillary ultrasound (12,13) to aid treatment planning such as the selection of appropriate axillary surgery (14), the initiation of neo-adjuvant systemic therapy (15) and reconstructive decisions for patients who may consequently benefit from post-mastectomy radiotherapy (16). The identification of SLN using microbubbles and CEUS was previously validated against the surgical detection of SLN using blue dye and radioisotope and concordance was found in 93% of cases that had a core biopsy (13). Other Centres in the UK also adopted the microbubble/ CEUS SLN biopsy technique for use in their own practice.

Previous work from Centre 1 has shown that patients with invasive breast cancer and a normal grey-scale ultrasound and benign microbubble/ CEUS SLN core biopsy are unlikely to have extensive metastatic axillary disease that is both grey-scale ultrasound occult and missed on SLN core biopsy (14). These results suggest that complete radiological staging of the axilla might be feasible.

We therefore aimed to assess the reproducibility of the microbubble/ CEUS SLN core biopsy procedure by comparing the test accuracy of Centre 1 with a smaller population of consecutive patients from Centre 2 as well as 2 other UK centres using patients, which were selected on the basis of tumour clinico-pathological features. We also examined the technical performance of individual radiologists at Centre 1. Lastly, we measured the volume of axillary metastases in patients from Centres 1 and 2 to determine the proportion of patients with a false negative microbubble/ CEUS SLN core biopsy who had 2 or more axillary LN macrometastases at the end of primary surgical treatment.

Methods

Study Design

Data was collected from 4 Breast Units across the UK (figure 1). In Centres 1 and 2, patients were included with newly diagnosed breast cancer and an initial grey-scale axillary ultrasound +/- biopsy of indeterminate/ abnormal LN (17) and only those with normal axillary imaging/ benign pathology proceeded to have a microbubble/ CEUS SLN core biopsy. At Centre 3, patients with newly diagnosed grade 2 or 3 invasive breast cancer and a normal grey-scale axillary ultrasound or indeterminate LN that were inadequate (not enough cells to make a diagnosis) or benign on cytology were included. At Centre 4, patients with newly diagnosed T2 or larger tumours, grade 2 or 3 invasive breast cancer with grey-scale ultrasound indeterminate axillary LN were included. Centres 3 and 4 selected patients at the discretion of the multi-disciplinary team. In all centres, pregnant patients and those with locally advanced/ metastatic disease were excluded. At Centre 1, 1361 consecutive patients were identified from a prospective database of patients having microbubble/ CEUS guided SLN core biopsy between December 2010 and November 2016. A proportion of this data (570 patients) has been previously analysed and published (14). Retrospective data was collected on 376 consecutive patients from Centre 2 between July 2010 and July 2014. Retrospective data was collected on 122 selected patients from Centre 3 between July 2013 and December 2015. Prospective data was collected on 48 selected patients from Centre 4 between June 2013 and February 2015.

Following a diagnosis of breast cancer, patients had an injection of ultrasound contrast agent (Sonovue, BRACCO Imaging S.p.A, Italy). 0.2ml of ultrasound contrast agent was injected intra-dermally in the periareolar, upper outer quadrant position using a 26G needle with a 1 ml tuberculin syringe at Centre 1, Centre 2 and Centre 3. Centre 4 used a 24G needle with a 1 ml tuberculin syringe. Lymphatic channels were visualised on contrast pulse sequencing and tracked into the axilla. Areas of contrast accumulation were also imaged with grey scale or live dual images (figure 2). Further injections of contrast up to 1ml and 3 consecutive injections together with injection site massage for 10-30 seconds were performed if progress of contrast through the lymphatic vessels was slow/ not immediately evident. Once identified, the SLN was biopsied using a core biopsy technique, Centres 1 and 2 (Achieve automatic biopsy device, Carefusion, San Diego, California, USA. 2-3x 14-16G), Centre 3 (Trucut biopsy device, San Diego, California, USA. 3-4x 14G) and Centre 4 (Trucut biopsy device. 3-4x 18G). Microbubble/ CEUS guided SLN core biopsies were performed by consultant breast radiologists; 7 at Centre 1, 4 at Centre 2, 2 at Centre 3 and a single consultant breast radiologist at Centre 4. Three of the 7 radiologists at Centre 1 also worked at Centre 2. Very rarely 2 SLN were visualised and both were biopsied at Centre 1, Centre 2 and Centre 3. At Centre 4, only the first enhancing SLN was biopsied. Centre 1 ultrasound examinations were performed with a Sequoia 512 Acuson (Siemens Medical Systems, Issaquah, Wash, USA) or LOGIQ 9 (GE Healthcare, Fairfield, CT, USA) using a linear transducer operating at 4.5 to 15MHz. Centre 2 ultrasound examinations were performed with a LOGIQ 9 using a linear transducer operating at 4.5 to 15MHz, Centre 3 ultrasound examinations

were performed with a Siemens Antaris or Acuson S1000 (Siemens Medical Systems, Issaquah, Wash, USA) and Centre 4 ultrasound examinations were performed with a Toshiba Aplio 500 (Toshiba Medical Systems Europe B.V., Zilverstraat 1, 2718RP, Zoetermeer, The Netherlands) using a mid-range linear probe (PLT-704SBT Linear). All ultrasound machines provided: conventional grey-scale, pulse-inversion harmonic grey scale, contrast specific sonographic imaging with live dual images of tissue only and contrast agent images. In order to reduce microbubble destruction, low mechanical index values were applied (MI: 0.1-0.4). Each centre performed histological analysis on biopsy samples. Centres 1 and 2 also used pancytokeratin (MNF116) immunohistochemical staining for patients with a lobular phenotype.

Surgical Management of the axilla

Initially, at Centre 1 and Centre 2 if the microbubble/ CEUS SLN core biopsy contained malignant cells, patients were advised to have an ipsilateral ALND. Later in the study period (Centre 1 and Centre 2) and throughout the study period at Centre 3 and Centre 4, to avoid surgical overtreatment, patients were recommended to have ALND only if a macrometastasis (>2mm) was seen in the core biopsy specimen and those with core biopsy isolated tumour cells (ITC) or micrometastases (<2mm) had SLNE. Patients also had SLNE if the core biopsy identified indeterminate cellular changes, normal lymphoid tissue, inadequate tissue sampling or the patient declined primary ALND. Before surgery, patients had an injection of radioactive isotope (Nanocoll, G.E Healthcare, Chicago, USA), between 20 and 40mbeq in the peri-areolar, upper outer quadrant position. Later that day or the following day, patients underwent surgical resection. After

anaesthetic induction, patients received a 2ml injection of blue dye (Bleu Patente V 2.5%, Guerbet, France) sub-dermally in the periareolar, upper outer quadrant position. A gamma probe (Navigator GPS, RMD Instruments, Watertown, USA) was used to identify SLN. All SLN within the axilla were excised and sent for local histological analysis including immunohistochemical staining for patients with a lobular phenotype at Centres 1 and 2. The total volume of axillary metastases at the end of surgical treatment was determined for each patient using the following scoring system: 1 (LN containing a macrometastasis), 0.5 (LN containing a micrometastasis) and 0.2 (LN containing isolated tumour cells)(12).

Statistics

The proportion of patients who had SLN identified was calculated. Using contingency tables, the sensitivity and specificity of a successful (LN tissue retrieved) CEUS guided SLN core biopsy, as the index test to identify SLN metastases in breast cancer patients with invasive disease was determined with axillary surgery (SLNE/ ALND) as the reference standard. The estimated values were calculated along with corresponding 95% confidence intervals (CI) illustrating the uncertainty in the results. All CI were calculated using the exact binomial method. The prevalence of SLN metastases in each population was derived from the reference standard. For centres 1 and 2, positive and negative predictive values were calculated directly, as they both employed consecutive enrolment so the study prevalence will be representative of the population of interest. For centres 3 and 4, which only included a non-randomly selected subset of eligible patients, Bayesian methods were used to calculate positive and negative predictive values, using the prevalence from centre 1 (the largest centre

with consecutive enrolment). The Mann-Whitney test was used to compare the volume of axillary metastases between patients with a false negative and true positive microbubble/ CEUS SLN biopsy. All analysis was completed using Stata software (Stata version 13.1; Stata Corp LP, College Station, Tx, USA).

Ethics

Kent Research Ethics Committee, UK approved the original trials at Centre 1 (reference numbers: 04/Q1801/25 and 11/H1101/1) and the Medicines and Healthcare Products Regulatory Agency (MHRA) sanctioned the use of microbubbles by intraparenchymal injection (Eudract Number: 2004-002423-41). All participating Centres were NHS Trusts and the New Procedures Committees at Centre 1, Centre 2, and Centre 3 and the Breast Services Committee at Centre 4 each approved the use of intradermal microbubbles and CEUS to identify and biopsy SLN in patients with breast cancer at their institution.

Results

Visualisation and targeted core biopsy of SLN using intradermal microbubbles and CEUS

A total of 1361 consecutive patients were identified from a prospectively maintained database at Centre 1. Complete data was not available for 12 patients and 1 patient had a fine needle aspiration biopsy (FNAB) rather than a core biopsy. Sentinel lymph nodes were clearly visualised by CEUS in 1216 of the 1348 patients (90%). In the 132 cases where the intradermally injected microbubbles failed to traffic through lymphatics to identify SLN, 25 (19%) had previous surgery on the breast/ axilla, a post breast biopsy haematoma or malignant involvement of the nipple areolar complex. A successful SLN core biopsy was achieved in 1083 of the 1348 patients (80%). The performance statistics of the 7 Centre 1 radiologists are presented in Table 1.

A total of 376 patients were identified retrospectively at Centre 2. Complete data was not available for 15 patients, 3 patients had a FNAB and 15 patients did not have the microbubbles/ CEUS procedure. Sentinel lymph nodes were clearly visualised by CEUS in 290 of 343 patients (84.5%). A successful core biopsy of SLN was achieved in 273 of 343 patients (79.6%).

At Centre 3, 121 patients were identified retrospectively. Complete data was not available for one patient. Sentinel lymph nodes were clearly visualised by CEUS in 109 of the 120 patients (90.8%). A successful core biopsy of SLN was achieved in 93 of 120 patients (77.5%). Prospective data was collected on 48 patients at Centre 4. There were technical problems with the initial 5 patients, as the lymphatics could not be visualised. However, once the settings on the ultrasound machine were optimised, SLN were clearly visualised and successfully core

biopsied in 38 of 43 patients (88%). In all centres, there were no allergic reactions following the administration of contrast agent and only one significant bleeding complication (Centre 1) of a large haematoma after SLN core biopsy which was evacuated 2 weeks later at the time of primary surgical treatment.

Identification of SLN metastases in patients with invasive breast cancer and a successful microbubble/ CEUS SLN core biopsy that underwent primary surgical treatment

In patients with a successful microbubble/ CEUS SLN core biopsy, the following were excluded from final analysis; those with pre-invasive disease (DCIS), those who had primary systemic or endocrine therapy and patients who did not proceed with axillary surgery because of choice or inability to tolerate a general anaesthetic. At Centres 1 and 2, patients with un-biopsied grey-scale abnormal LN were excluded and 6 patients at Centre 1 were also excluded because of incomplete surgical data.

At Centre 1, 816 of the 1083 patients with a successful microbubble/ CEUS SLN core biopsy went on to have primary surgical treatment. Evidence of a core biopsy tract was seen in the excised SLN of 656 (80%) patients, 127 (16%) patients did not have a core biopsy tract visualized in excised SLN, 2 (0.2%) patients had a core biopsy tract seen in non-SLN and data regarding the presence of a core biopsy tract was not recorded for 31 (3.8%) patients. For the other centres; 215 of 273 patients (Centre 2), 80 of 93 patients (Centre 3) and 38 of 38 patients (Centre 4) with a successful microbubble/ CEUS SLN core biopsy went on to have primary surgical treatment. Comprehensive data documenting evidence of previous biopsy in excised SLN was not available for Centres 2, 3 and

4. For each centre, the accuracy of a successful microbubble/ CEUS SLN core biopsy to identify SLN metastases in patients with invasive breast cancer is presented in Table 2.

Volume of axillary metastases at the end of primary surgical treatment in patients with invasive breast cancer, radiologically normal axillary LN and a successful microbubble/ CEUS SLN core biopsy in Centres 1 and 2

At Centre 1, 95 patients had a false negative microbubble/ CEUS SLN core biopsy with metastases found in the excised SLN. Of these, evidence of a core biopsy tract was seen in the excised SLN of 69 (72.6%) patients, 23 (24.2%) patients did not have a core biopsy tract visualised in excised SLN and data regarding the presence of a core biopsy tract was not recorded for 3 (3.2%) patients. Sixty-one patients (64%) with a false negative microbubble/ CEUS SLN core biopsy result went on to have a completion ALND and 34 (36%) had no further axillary surgery (axillary conservation). At the end of primary surgical treatment, 12 patients (12.6%) were found to have 2 or more LN macrometastases and 82 patients (87.4%) had less than 2 LN macrometastases. Of the 730 patients with an initial benign microbubble/ CEUS SLN core biopsy, only 12 (1.6%) had 2 or more LN macrometastases found at the end of primary surgical treatment (Table 3). Eighty-four patients at Centre 1 had a true positive microbubble/ CEUS SLN core biopsy and 81 (96%) had an ALND whereas 3 (4%) had a SLNE. Of these 84 patients, 42 (50%) had 2 or more axillary LN macrometastases at the end of primary surgical treatment.

At Centre 2, 28 patients had a false negative microbubble/ CEUS SLN core biopsy with metastases found in the excised SLN. Nineteen patients (68%) went on to

have a completion ALND and 9 (32%) had axillary conservation. At the end of primary surgical treatment, 7 patients (25%) were found to have 2 or more axillary macrometastases and 22 patients (75%) had a malignant axillary LN score less than 2 or had ITC in multiple LN (one patient with ITC in 13 LN). Of the 181 patients with an initial benign microbubble/ CEUS SLN core biopsy, 7 (4%) had 2 or more axillary macrometastases found at the end of primary surgical treatment (Table 4). Thirty-one patients at Centre 2 had a true positive microbubble/ CEUS SLN core biopsy and 30 (97%) had an ALND whereas 1 (3%) had a SLNE. Of these 31 patients, 14 (45%) had 2 or more axillary LN macrometastases at the end of primary surgical treatment.

For both centres, at the end of surgical treatment the difference between the final malignant LN score of false negative versus true positive microbubble/ CEUS SLN core biopsies was statistically significant (figure 3).

Discussion

Despite the use of different ultrasound machines and variations in the methods and patient selection, the identification and core biopsy of SLN using intradermal microbubbles and CEUS in patients with breast cancer is a reproducible technique across multiple centres. Overall, the visualisation of SLN across the 4 centres ranged from 84.5% to 90.8% and a successful core biopsy from 77.5% to 88%. Factors that appeared to adversely affect the visualisation of SLN at Centre 1 included previous surgery and disease involvement of the nipple areolar complex.

There is undoubtedly a learning curve associated with the procedure and familiarity with the equipment is important as demonstrated by the data from

Centre 4 where they experienced technical problems with the first 5 patients. The performance of Centre 1 radiologists also highlight the distinct competencies of the 2 components of the procedure, namely SLN identification and SLN core biopsy. Even though identical ultrasound equipment and methods were used, the percentage of procedures with successful visualisation of SLN varied from 97% to 73%, which suggests either that not all radiologists at Centre 1 received adequate procedural training before performing the test or some found it difficult to visualise microbubbles trafficking through lymphatic channels. Six of the 7 radiologists were fairly consistent in their ability to successfully core biopsy visualised SLN, but 1 obviously struggled and only successfully retrieved lymphoid tissue in 70% of cases. Anecdotally, most radiologists accustomed to the procedure recommend that novices observe 3 cases, then perform 10 cases supervised before undertaking 30 independent procedures with an audit of their results. Once proficient, the whole procedural time is 15 to 30 minutes.

There is scope to improve the technology of CEUS. In swine models, LN metastases can be identified as areas devoid of contrast agent (11) and in a recent study of breast cancer patients, the sensitivity of CEUS as a test to identify SLN metastases using only enhancement patterns (no biopsy) was 81.8% (18). Innovations such as ultrafast ultrasound (19), super resolution imaging (20) and improved lymphatic microbubble transit (21) may improve the ability of clinicians to visualise SLN and achieve a reliable standard.

The sensitivity of a microbubble/ CEUS core biopsy as a test to identify SLN metastases in patients with invasive breast cancer and a normal grey-scale axillary ultrasound/ benign axillary LN biopsy is consistently around 50% with

Centres 2, 3 and 4 within the 95% confidence intervals of Centre 1. As grey-scale axillary ultrasound can usefully identify approximately 50% of LN metastases (22), the addition of a microbubble/ CEUS SLN core biopsy substantially increases the overall detection rate for metastatic axillary LN. Consequently, the negative predictive value of the test is high and <5% of patients (Centres 1 and 2) with a normal grey-scale ultrasound and benign microbubble/ CEUS SLN core biopsy had 2 or more LN macrometastases detected by axillary surgery.

We have previously speculated that the technique has a high false negative rate because the core biopsy fails to pick up small metastatic deposits in SLN (13) and this may be the reason why very few patients with a false negative microbubble/ CEUS core biopsy at Centres 1 and 2 had two or more axillary LN macrometastases found at the end of primary surgical treatment. Yet, retrieving more LN tissue with a vacuum-assisted biopsy technique does not appear to appreciably increase the sensitivity of microbubbles and CEUS (23).

Alternatively, it is usual for only one SLN to be visualised and biopsied with CEUS but the median number of SLN retrieved with a surgical excision is 2 (3). In this series, 80% of surgically excised LN at Centre 1 showed evidence of a previous core biopsy and this proportion dropped to 72.6% in patients with a false negative benign microbubble/ CEUS SLN core biopsy. This raises the possibility that in the false negative cases, the second or subsequent SLN contained the metastases and perhaps more than one SLN should be actively sought with the microbubbles/ CEUS procedure.

When compared to Centre 1, a higher proportion of Centre 2 patients with a benign microbubble/ CEUS SLN core biopsy had two or more axillary macrometastases found at the end of surgical treatment (1.8 vs 4%). This may

be related to the smaller patient sample size in Centre 2 or the higher prevalence of LN metastases in patients with a successful microbubble/ CEUS SLN core biopsy in Centre 2 (27% vs 22% at Centre 1). As the patient and clinicopathologic features of the tumours were similar in both centres, the greater prevalence of LN metastases at Centre 2 may be a consequence of a lower initial metastatic LN detection rate with grey-scale axillary ultrasound.

The difference in the volume of axillary disease between patients with a false negative and true positive microbubble/ CEUS SLN core biopsy at the end of primary surgical treatment was statistically significant in Centres 1 and 2. When compared to the false negative groups in Centres 1 and 2, more patients with a true positive microbubble/ CEUS SLN core biopsy had complete axillary surgery (ALND) rather than axillary conservation (SLNE). The retrieval of a greater number of LN in the true positive groups from Centres 1 and 2 may therefore have increased the total number of axillary LN metastases found at the end of surgical treatment and influenced the final metastatic score. However, in a previous publication from Centre 1 (14) comparing only patients with complete axillary surgery (ALND), the difference in the volume of axillary disease between those with a false negative and true positive microbubble/ CEUS SLN core biopsy remained stastically significant.

An argument against using CEUS to biopsy SLN in routine practice is that patients with a biopsy containing malignant cells are committed to a primary ALND for what may be a low burden of axillary disease. This can be mitigated against by offering SLNE to patients with micrometastases in the core biopsy specimen. It should also be noted that 50% of patients at Centre 1 and 45% of patients at Centre 2 with a true positive microbubble/ CEUS SLN core biopsy had

2 or more axillary macrometastases found at the end of surgical treatment and therefore using the test for patients who are not eligible for axillary conservation (7) is beneficial.

Conclusion

The results of the American College of Surgeons Oncology Group Z0011 trial (6) have changed practice by showing that loco-regional control of axillary metastases is not solely dependant upon surgical excision and residual disease can be treated with adjuvant therapy. In addition, anatomic staging of breast cancer is likely to become less relevant to treatment decisions as tumour genomic and molecular assays are better understood (24). Based on recent information obtained from Centre 1, a SLNE costs 3.6x the cost of a microbubble/ CEUS SLN core biopsy (£671.63 vs £189 respectively). Omitting axillary surgery will improve theatre utilisation (potentially allowing more cases to be added to a list) as well as reducing the anaesthetic time for each patient. In the era of axillary conservation, molecular medicine and dwindling resources the combination of grey-scale axillary ultrasound and microbubble/ CEUS SLN core biopsy has the potential to succeed surgical staging of the axilla. Further work now needs to be undertaken to refine the procedure with protocols, standard setting and training.

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Figure Captions

Figure 1

Diagram showing the selection and flow of participants from each centre through the study. Lymph node (LN), Sentinel Lymph Node (SLN), Contrast enhanced ultrasound (CEUS), Ductal Carcinoma in situ (DCIS), isolated tumour cells (ITC), surgical sentinel lymph node biopsy (SLNB), axillary lymph node dissection (ALND) and POSNOC – (POsitive Sentinel NOde: adjuvant therapy alone versus adjuvant therapy plus Clearance or axillary radiotherapy. A randomised controlled trial of axillary treatment in women with early stage breast cancer who have metastases in one or two sentinel nodes).

Figure 2

A. Ultrasound contrast pulse sequencing image of a SLN (white arrow) after injection of intradermal microbubbles (between 0.2 and 1 ml injected using a 26G needle with 1ml tuberculin syringe) into the UOQ periareolar area of the breast. B. Grey scale ultrasound image of the same SLN as visualised in A. Images provided by Centre 3.

Figure 3

A. Volume of axillary disease at the end of primary surgical treatment for individual patients at Centres 1 and 2 who had a false negative (FN) or true positive (TP) microbubble/ CEUS core biopsy of SLN. Axillary lymph

node dissection (ALND) and sentinel lymph node excision (SLNE). B. The total volume of axillary metastases at the end of surgical treatment was determined using a scoring system (isolated tumour cells = 0.2, each LN micrometastasis = 0.5 and each LN macrometastasis = 1). The Mann-Whitney test was used to compare the volume of axillary metastases between patients with a false negative and true positive microbubble/ CEUS SLN biopsy in both centres.

Table 1

Performance statistics of 7 consultant breast radiologists at Centre 1 (Total 1349 of 1361 procedures). In 12 cases the data was incomplete and the name of the radiologist was not recorded. For radiologist no.1, one successful procedure was a fine needle aspiration biopsy rather than a core biopsy.

Table 2

Test accuracy of CEUS guided SLN biopsy using intradermally injected microbubbles in women with normal (centres 1 and 2) or indeterminate results from previous grey scale ultrasound (with or without previous biopsy). Reference standard is Sentinel Lymph node excision (SLNE) or axillary lymph node dissection (ALND). Positive predictive value (PPV) and negative predictive value (NPV) were calculated directly for centres 1 and 2 who employed consecutive recruitment, and using Bayesian methods with 22% prevalence at centres 3 and 4 as these were not a

consecutively or randomly selected group. True positive (TP), false positive (FP), true negative (TN) and false negative (FN).

Table 3

Age and clinicopathological characteristics of all patients at Centre 1 (first column) with a successful microbubble/CEUS SLN core biopsy before primary surgical treatment and Centre 1 patients with false negative microbubble/ CEUS SLN core biopsies sub-divided into micrometastases (<2mm)/ isolated tumour cells (ITC), low volume metastases and high volume metastases identified at the end of primary surgical treatment. Data are expressed as *n* (%). Estrogen receptor (ER), progesterone receptor (PR), human epidermal growth factor receptor 2 (Her2), invasive ductal carcinoma (IDC), invasive lobular carcinoma (ILC).

Table 4

Age and clinicopathological characteristics of all patients at Centre 2 (first column) with a successful microbubble/CEUS SLN core biopsy before primary surgical treatment and Centre 2 patients with false negative microbubble/ CEUS SLN core biopsies sub-divided into micrometastases (<2mm)/ isolated tumour cells (ITC), low volume metastases and high volume metastases identified at the end of primary surgical treatment. Data are expressed as *n* (%). Estrogen receptor (ER), progesterone receptor (PR), human epidermal growth factor receptor 2 (Her2), invasive ductal carcinoma (IDC), invasive lobular carcinoma (ILC).

Table 1

	Centre 1 – Consultant Breast Radiologists						
	1	**2**	**3**	**4**	**5**	**6**	**7**
Total Procedures	276	37	501	81	116	207	131
Procedures where core biopsy not attempted	0 (0%)	0 (0%)	0 (0%)	2 (2 .5%)	0 (0 %)	0 (0 %)	1 (0.8%)
Procedures with successful visualisation of SLN	269 (97.5%)	33 (89.2%)	457 (91.2%)	59 (72.8%)	94 (81%)	187 (90.3%)	118 (90.1%)
Procedures without SLN tissue retrieved	1 (0.4%)	1 (2.7%)	64 (12.8%)	2 (2.5%)	10 (8.6%)	18 (8.7%)	34 (26%)
Successful retrieval of lymphoid tissue in those with visualised SLN	268 (99.6%)	32 (97%)	393 (86%)	57 (96.6%)	84 (89.4%)	169 (90.4%)	84 (71.2%)
Total successful visualisation and core biopsy of SLN	268 (97.1%)	32 (86.5%)	393 (78.4%)	57 (70.4%)	84 (72.4%)	169 (81.6%)	84 (64.1%)

Table 2

Centre	Prevalence of LN metastases	TP	FP	TN	FN	Sensitivity	Specificity	PPV	NPV
1	22%	84	2	635	95	46.9% (39.4-55.5%)	99.7 % (98.95-100%)	97.7% (91.9-99.7%)	87.0% (84.3-89.3%
2	27%	31	3	153	28	52.5% (39.1-65.7%)	98.1% (94.5-99.6%)	91.2% (76.3-98.1%)	84.5% (78.4-89.5%)
3	35%	13	0	52	15	46.4% (27.5-66.1%)	100% (93.2-100%)	100% (75.3-100%)	86.9% (82.4-90.3%)
4	29%	5	1	26	6	45.5% (16.7-76.6%)	96.3% (81.0-99.9%)	77.6% (31.3-96.3%)	86.2 % (78.4 -91.5%)

Table 3

		Volume of disease at the end of surgical treatment (microbubble/ CEUS false negative core biopsy)		
Centre 1	**All Patients**	**Micrometastasis/ ITC**	**Low (<2 LN macrometastases)**	**High (2 or more LN macrometastases)**
Total number of patients	816	37	46	12
		15 (41%) ALND	34 (74%) ALND	12 (100%) ALND
Median age in years (range)	61 (30-94)	61 (42-89)	55 (32-90)	53 (36-69)
Receptor Status				
ER positive	707 (87%)	32 (86%)	40 (87%)	11 (92%)
ER unknown	4 (0.4%)	0	0	0
Her-2 positive	79 (10%)	3 (8%)	2 (4%)	1 (8%)
Her-2 not recorded	13 (1.6%)	0	2 (4%)	0
ER-/PR-/Her-2-	72 (10%)	4 (10.8%)	4 (9%)	0
ER-/PR-/HER-2+	28 (3%)	1 (2.7%)	1 (2%)	1 (8%)
Invasive Tumour Size				
DCIS + microinvasion	5 (0.6%)	0	0	0
T1	472 (58%)	19 (51%)	17 (37%)	4 (33%)
T2	199 (24%)	10 (27%)	18 (39%)	5 (42%)
T3	29 (4%)	5 (14%)	3 (7%)	1 (8%)
Multifocal	111 (14%)	3 (8%)	8 (17%)	2 (17%)
Unknown	0	0	0	0
Tumour Grade				
Grade 1	180 (22%)	6 (16%)	7 (15%)	4 (33%)
Grade 2	392 (48%)	20 (54%)	21 (46%)	6 (50%)
Grade 3	209 (26%)	11 (30%)	14 (30%)	1 (8%)
Mixed grade	29 (4%)	0	4 (9%)	1 (8%)
Unknown	1 (0.1%)	0	0	0
Tumour Type				
IDC	660 (81%)	23 (62%)	38 (83%)	11 (92%)
ILC	103 (13%)	11 (30%)	6 (13%)	1 (8%)
Other	29 (4%)	1 (3%)	1 (2%)	0
Mixed	17 (2%)	2 (5%)	1 (2%)	0
Unknown	2 (0.2%)	0	0	0

Table 4

		Volume of disease at the end of surgical treatment (microbubble/ CEUS false negative core biopsy)		
Centre 2	**All Patients**	**Micrometastasis/ ITC**	**Low (<2 LN macrometastases)**	**High (2 or more LN macrometastases)**
Total number of patients	215	10	11	7
		2 (20%) ALND	10 (91%) ALND	7 (100%) ALND
Median age in years (range)	64 (31-93)	63.5 (37-93)	62 (38-91)	55 (47-72)
Receptor Status				
ER positive	176 (82%)	10 (100%)	8 (73%)	6 (86%)
ER unknown	6 (3%)	0	1 (9%)	1 (14%)
Her-2 positive	29 (13%)	1 (10%)	0	1 (14%)
Her-2 not recorded	8 (4%)	0	1 (9%)	1 (14%)
ER-/PR-/Her-2-	23 (11%)	0	1 (9%)	0
ER-/PR-/HER-2+	10 (5%)	0	0	0
Invasive Tumour Size				
DCIS + microinvasion	0	0	0	0
T1	118 (55%)	2 (20%)	6 (55%)	2 (29%)
T2	71 (33%)	6 (60%)	4 (36%)	5 (71%)
T3	9 (4%)	0	0	0
Multifocal	17 (8%)	2 (20%)	0	0
Unknown	0	0	1 (9%)	0
Tumour Grade				
Grade 1	35(16%)	1 (10%)	0	2 (29%)
Grade 2	102 (47%)	6 (60%)	5 (45.5%)	1 (14%)
Grade 3	68 (32%)	2 (20%)	4 (36%)	4 (57%)
Mixed grade	4 (2%)	0	0	0
Unknown	6 (3%)	0	2 (18%)	0
Tumour Type				
IDC	160 (74%)	7 (70%)	8 (73%)	5 (71%)
ILC	24 (11%)	2 (20%)	1 (9%)	0
Other	16 (7%)	0	0	0
Mixed	13 (6%)	1 (10%)	1 (9%)	2 (29%)
Unknown	2 (1%)	0	1 (9%)	0

Enhanced pre-operative axillary staging using intradermal microbubbles and contrast-enhanced ultrasound to detect and biopsy sentinel lymph nodes in breast cancer: A potential replacement for axillary surgery

Short title: Microbubble and CEUS core biopsy of sentinel nodes in breast cancer

Abstract

Objectives

To compare the experience of 4 UK Centres in the use of intradermal microbubbles and contrast enhanced ultrasound (CEUS) to pre-operatively identify and biopsy sentinel lymph nodes (SLN) in patients with breast cancer.

Methods

In all centres, breast cancer patients had a microbubble/ CEUS SLN core biopsy prior to axillary surgery and patients in Centres 1 and 2 had a normal grey-scale axillary ultrasound. Data was collected between 2010 and 2016; 1361 from Centre 1 (prospective, sequential), 376 from Centre 2 (retrospective, sequential), 122 from Centre 3 (retrospective, selected) and 48 from Centre 4 (prospective, selected).

Results

SLN were successfully core biopsied in 80% (Centre 1), 79.6% (Centre 2), 77.5% (Centre 3) and 88% (Centre 4). The sensitivities to identify all SLN metastases were 46.9% (95% CI 39.4-55.1), 52.5% (95% CI 39.1-65.7), 46.4% (95% CI 27.5-66.1) and 45.5% (95% CI 16.7-76.6) respectively. Specificities; 99.7% (95% CI 98.9-100), 98.1% (95% CI 94.5-99.6), 100% (95% CI 93.2-100%) and 96.3%

(95% CI 81-99.9) respectively. Negative predictive values; 87.0% (95% CI 84.3-89.3), 84.5% (95% CI 78.4-89.5), 86.9% (95% CI 82.4-90.3) and 86.2% (95% CI 78.4-91.5) respectively. At Centres 1 and 2, 12/730 (1.6%) and 7/181 (4%) respectively of patients with a benign microbubble/ CEUS SLN core biopsy had 2 or more lymph node (LN) macrometastases found at the end of primary surgical treatment.

Conclusions

The identification and biopsy of SLN using CEUS is a reproducible technique.

Advances in knowledge

In the era of axillary conservation, microbubble/ CEUS SLN core biopsy has the potential to succeed surgical staging of the axilla.

Introduction

Sentinel lymph node excision (SLNE) is the axillary staging method of choice for breast cancer patients with normal axillary lymph nodes (LN) on grey-scale ultrasound or a benign biopsy of morphologically abnormal LN (1,2). Although SLNE has less reported morbidity than axillary lymph node dissection (ALND) it remains a surgical procedure performed under general anaesthesia with recognised immediate complications such as infection (11%) and long term problems with sensory loss (11%) and arm lymphoedema (5%) at 12 months (3) The operation is also reliant upon 2 tracers (radioactive isotope and blue dye) to locate SLN and maintain a false negative rate of 6% (4). The blue dye carries a hazard of anaphylaxis (0.9%) (5) and there are logistical challenges in obtaining medical grade radioisotopes.

Recently, the practice of removing all malignant axillary LN to achieve local control has been challenged by the results of a trial where patients with SLN metastases were randomized to a completion ALND or no further axillary surgery (6). The local recurrence rate in the axilla was low with no difference between the groups despite the fact that 27.3% of patients in the ALND arm had further lymph node metastases retrieved (6). These results emphasize the role of modern adjuvant treatment in preventing loco-regional~~al~~ disease recurrence and have led to the introduction of conservative surgical management of the axilla for patients with up to 2 SLN macrometastases (7).

In patients with breast cancer, SLN can be identified and percutaneously biopsied in the clinic using intradermally injected microbubbles and contrast-enhanced ultrasound (CEUS) (8, 9, 10). The technique was originally described in a swine melanoma model (11) and following a trial period, was incorporated

into routine practice at Centre 1 for all invasive breast cancer patients with a normal grey-scale axillary ultrasound (12,13) to aid treatment planning such as the selection of appropriate axillary surgery (14), the initiation of neo-adjuvant systemic therapy (15) and reconstructive decisions for patients who may consequently benefit from post-mastectomy radiotherapy (16). The identification of SLN using microbubbles and CEUS was previously validated against the surgical detection of SLN using blue dye and radioisotope and concordance was found in 93% of cases that had a core biopsy (13). Other Centres in the UK also adopted the microbubble/ CEUS SLN biopsy technique for use in their own practice.

Previous work from Centre 1 has shown that patients with invasive breast cancer and a normal grey-scale ultrasound and benign microbubble/ CEUS SLN core biopsy are unlikely to have extensive metastatic axillary disease that is both grey-scale ultrasound occult and missed on SLN core biopsy (14). These results suggest that complete radiological staging of the axilla might be feasible.

We therefore aimed to assess the reproducibility of the microbubble/ CEUS SLN core biopsy procedure by comparing the test accuracy of Centre 1 with a smaller population of consecutive patients from Centre 2 as well as 2 other UK centres using patients, which were selected on the basis of tumour clinico-pathological features. We also examined the technical performance of individual radiologists at Centre 1. Lastly, we measured the volume of axillary metastases in patients from Centres 1 and 2 to determine the proportion of patients with a false negative microbubble/ CEUS SLN core biopsy who had 2 or more axillary LN macrometastases at the end of primary surgical treatment.

Methods

Study Design

Data was collected from 4 Breast Units across the UK (figure 1). In Centres 1 and 2, patients were included with newly diagnosed breast cancer and an initial grey-scale axillary ultrasound +/- biopsy of indeterminate/ abnormal LN (17) and only those with normal axillary imaging/ benign pathology proceeded to have a microbubble/ CEUS SLN core biopsy. At Centre 3, patients with newly diagnosed grade 2 or 3 invasive breast cancer and a normal grey-scale axillary ultrasound or indeterminate LN that were inadequate (not enough cells to make a diagnosis) or benign on cytology were included. At Centre 4, patients with newly diagnosed T2 or larger tumours, grade 2 or 3 invasive breast cancer with grey-scale ultrasound indeterminate axillary LN were included. Centres 3 and 4 selected patients at the discretion of the multi-disciplinary team. In all centres, pregnant patients and those with locally advanced/ metastatic disease were excluded. At Centre 1, 1361 consecutive patients were identified from a prospective database of patients having microbubble/ CEUS guided SLN core biopsy between December 2010 and November 2016. A proportion of this data (570 patients) has been previously analysed and published (14~~reference blinded for review~~). Retrospective data was collected on 376 consecutive patients from Centre 2 between July 2010 and July 2014. Retrospective data was collected on 122 selected patients from Centre 3 between July 2013 and December 2015. Prospective data was collected on 48 selected patients from Centre 4 between June 2013 and February 2015.

Identification and biopsy of SLN using intradermal microbubbles and CEUS

Following a diagnosis of breast cancer, patients had an injection of ultrasound contrast agent (Sonovue, BRACCO Imaging S.p.A, Italy). 0.2ml of ultrasound contrast agent was injected intra-dermally in the periareolar, upper outer quadrant position using a 26G needle with a 1 ml tuberculin syringe at Centre 1, Centre 2 and Centre 3. Centre 4 used a 24G needle with a 1 ml tuberculin syringe. Lymphatic channels were visualised on contrast pulse sequencing and tracked into the axilla. Areas of contrast accumulation were also imaged with grey scale or live dual images (figure 2). Further injections of contrast up to 1ml and 3 consecutive injections together with injection site massage for 10-30 seconds were performed if progress of contrast through the lymphatic vessels was slow/ not immediately evident. Once identified, the SLN was biopsied using a core biopsy technique, Centres 1 and 2 (Achieve automatic biopsy device, Carefusion, San Diego, California, USA. 2-3x 14-16G), Centre 3 (Trucut biopsy device, San Diego, California, USA. 3-4x 14G) and Centre 4 (Trucut biopsy device. 3-4x 18G). Microbubble/ CEUS guided SLN core biopsies were performed by consultant breast radiologists; 7 at Centre 1, 4 at Centre 2, 2 at Centre 3 and a single consultant breast radiologist at Centre 4. Three of the 7 radiologists at Centre 1 also worked at Centre 2. Very rarely 2 SLN were visualised and both were biopsied at Centre 1, Centre 2 and Centre 3. At Centre 4, only the first enhancing SLN was biopsied. Centre 1 ultrasound examinations were performed with a Sequoia 512 Acuson (Siemens Medical Systems, Issaquah, Wash, USA) or LOGIQ 9 (GE Healthcare, Fairfield, CT, USA) using a linear transducer operating at 4.5 to 15MHz. Centre 2 ultrasound examinations were performed with a LOGIQ 9 using

a linear transducer operating at 4.5 to 15MHz, Centre 3 ultrasound examinations were performed with a Siemens Antaris or Acuson S1000 (Siemens Medical Systems, Issaquah, Wash, USA) and Centre 4 ultrasound examinations were performed with a Toshiba Aplio 500 (Toshiba Medical Systems Europe B.V., Zilverstraat 1, 2718RP, Zoetermeer, The Netherlands) using a mid-range linear probe (PLT-704SBT Linear). All ultrasound machines provided: conventional grey-scale, pulse-inversion harmonic grey scale, contrast specific sonographic imaging with live dual images of tissue only and contrast agent images. In order to reduce microbubble destruction, low mechanical index values were applied (MI: 0.1-0.4). Each centre performed histological analysis on biopsy samples. Centres 1 and 2 also used pancytokeratin (MNF116) immunohistochemical staining for patients with a lobular phenotype.

Surgical Management of the axilla

Initially, at Centre 1 and Centre 2 if the microbubble/ CEUS SLN core biopsy contained malignant cells, patients were advised to have an ipsilateral ALND. Later in the study period (Centre 1 and Centre 2) and throughout the study period at Centre 3 and Centre 4, to avoid surgical overtreatment, patients were recommended to have ALND only if a macrometastasis (>2mm) was seen in the core biopsy specimen and those with core biopsy isolated tumour cells (ITC) or micrometastases (<2mm) had SLNE. Patients also had SLNE if the core biopsy identified indeterminate cellular changes, normal lymphoid tissue, inadequate tissue sampling or the patient declined primary ALND. Before surgery, patients had an injection of radioactive isotope (Nanocoll, G.E Healthcare, Chicago, USA), between 20 and 40mbeq in the peri-areolar, upper outer quadrant position.

Later that day or the following day, patients underwent surgical resection. After anaesthetic induction, patients received a 2ml injection of blue dye (Bleu Patente V 2.5%, Guerbet, France) sub-dermally in the periareolar, upper outer quadrant position. A gamma probe (Navigator GPS, RMD Instruments, Watertown, USA) was used to identify SLN. All SLN within the axilla were excised and sent for local histological analysis including immunohistochemical staining for patients with a lobular phenotype at Centres 1 and 2. The total volume of axillary metastases at the end of surgical treatment was determined for each patient using the following scoring system: 1 (LN containing a macrometastasis), 0.5 (LN containing a micrometastasis) and 0.2 (LN containing isolated tumour cells)(12).

Statistics

The proportion of patients who had SLN identified was calculated. Using contingency tables, the sensitivity and specificity of a successful (LN tissue retrieved) CEUS guided SLN core biopsy, as the index test to identify SLN metastases in breast cancer patients with invasive disease was determined with axillary surgery (SLNE/ ALND) as the reference standard. The estimated values were calculated along with corresponding 95% confidence intervals (CI) illustrating the uncertainty in the results. All CI were calculated using the exact binomial method. The prevalence of SLN metastases in each population was derived from the reference standard. For centres 1 and 2, positive and negative predictive values were calculated directly, as they both employed consecutive enrolment so the study prevalence will be representative of the population of interest. For centres 3 and 4, which only included a non-randomly selected subset of eligible patients, Bayesian methods were used to calculate positive and

negative predictive values, using the prevalence from centre 1 (the largest centre with consecutive enrolment). The Mann-Whitney test was used to compare the volume of axillary metastases between patients with a false negative and true positive microbubble/ CEUS SLN biopsy. All analysis was completed using Stata software (Stata version 13.1; Stata Corp LP, College Station, Tx, USA).

Ethics

Kent Research Ethics Committee, UK approved the original trials at Centre 1 (reference numbers: 04/Q1801/25 and 11/H1101/1) and the Medicines and Healthcare Products Regulatory Agency (MHRA) sanctioned the use of microbubbles by intraparenchymal injection (Eudract Number: 2004-002423-41). All participating Centres were NHS Trusts and the New Procedures Committees at Centre 1, Centre 2, and Centre 3 and the Breast Services Committee at Centre 4 each approved the use of intradermal microbubbles and CEUS to identify and biopsy SLN in patients with breast cancer at their institution.

Results

Visualisation and targeted core biopsy of SLN using intradermal microbubbles and CEUS

A total of 1361 consecutive patients were identified from a prospectively maintained database at Centre 1. Complete data was not available for 12 patients and 1 patient had a fine needle aspiration biopsy (FNAB) rather than a core biopsy. Sentinel lymph nodes were clearly visualised by CEUS in 1216 of the 1348 patients (90%). In the 132 cases where the intradermally injected microbubbles failed to traffic through lymphatics to identify SLN, 25 (19%) had previous surgery on the breast/ axilla, a post breast biopsy haematoma or malignant involvement of the nipple areolar complex. A successful SLN core biopsy was achieved in 1083 of the 1348 patients (80%). The performance statistics of the 7 Centre 1 radiologists are presented in Table 1.

A total of 376 patients were identified retrospectively at Centre 2. Complete data was not available for 15 patients, 3 patients had a FNAB and 15 patients did not have the microbubbles/ CEUS procedure. Sentinel lymph nodes were clearly visualised by CEUS in 290 of 343 patients (84.5%). A successful core biopsy of SLN was achieved in 273 of 343 patients (79.6%).

At Centre 3, 121 patients were identified retrospectively. Complete data was not available for one patient. Sentinel lymph nodes were clearly visualised by CEUS in 109 of the 120 patients (90.8%). A successful core biopsy of SLN was achieved in 93 of 120 patients (77.5%). Prospective data was collected on 48 patients at Centre 4. There were technical problems with the initial 5 patients, as the lymphatics could not be visualised. However, once the settings on the ultrasound

machine were optimised, SLN were clearly visualised and successfully core biopsied in 38 of 43 patients (88%). In all centres, there were no allergic reactions following the administration of contrast agent and only one significant bleeding complication (Centre 1) of a large haematoma after SLN core biopsy which was evacuated 2 weeks later at the time of primary surgical treatment.

Identification of SLN metastases in patients with invasive breast cancer and a successful microbubble/ CEUS SLN core biopsy that underwent primary surgical treatment

In patients with a successful microbubble/ CEUS SLN core biopsy, the following were excluded from final analysis; those with pre-invasive disease (DCIS), those who had primary systemic or endocrine therapy and patients who did not proceed with axillary surgery because of choice or inability to tolerate a general anaesthetic. At Centres 1 and 2, patients with un-biopsied grey-scale abnormal LN were excluded and 6 patients at Centre 1 were also excluded because of incomplete surgical data.

At Centre 1, 816 of the 1083 patients with a successful microbubble/ CEUS SLN core biopsy went on to have primary surgical treatment. Evidence of a core biopsy tract was seen in the excised SLN of 656 (80%) patients, 127 (16%) patients did not have a core biopsy tract visualized in excised SLN, 2 (0.2%) patients had a core biopsy tract seen in non-SLN and data regarding the presence of a core biopsy tract was not recorded for 31 (3.8%) patients. For the other centres; 215 of 273 patients (Centre 2), 80 of 93 patients (Centre 3) and 38 of 38 patients (Centre 4) with a successful microbubble/ CEUS SLN core biopsy went on to have primary surgical treatment. Comprehensive data documenting

evidence of previous biopsy in excised SLN was not available for Centres 2, 3 and 4. For each centre, the accuracy of a successful microbubble/ CEUS SLN core biopsy to identify SLN metastases in patients with invasive breast cancer is presented in Table 2.

Volume of axillary metastases at the end of primary surgical treatment in patients with invasive breast cancer, radiologically normal axillary LN and a successful microbubble/ CEUS SLN core biopsy in Centres 1 and 2

At Centre 1, 95 patients had a false negative microbubble/ CEUS SLN core biopsy with metastases found in the excised SLN. Of these, evidence of a core biopsy tract was seen in the excised SLN of 69 (72.6%) patients, 23 (24.2%) patients did not have a core biopsy tract visualised in excised SLN and data regarding the presence of a core biopsy tract was not recorded for 3 (3.2%) patients. Sixty-one patients (64%) with a false negative microbubble/ CEUS SLN core biopsy result went on to have a completion ALND and 34 (36%) had no further axillary surgery (axillary conservation). At the end of primary surgical treatment, 12 patients (12.6%) were found to have 2 or more LN macrometastases and 82 patients (87.4%) had less than 2 LN macrometastases. Of the 730 patients with an initial benign microbubble/ CEUS SLN core biopsy, only 12 (1.6%) had 2 or more LN macrometastases found at the end of primary surgical treatment (Table 3). Eighty-four patients at Centre 1 had a true positive microbubble/ CEUS SLN core biopsy and 81 (96%) had an ALND whereas 3 (4%) had a SLNE. Of these 84 patients, 42 (50%) had 2 or more axillary LN macrometastases at the end of primary surgical treatment.

At Centre 2, 28 patients had a false negative microbubble/ CEUS SLN core biopsy with metastases found in the excised SLN. Nineteen patients (68%) went on to have a completion ALND and 9 (32%) had axillary conservation. At the end of primary surgical treatment, 7 patients (25%) were found to have 2 or more axillary macrometastases and 22 patients (75%) had a malignant axillary LN score less than 2 or had ITC in multiple LN (one patient with ITC in 13 LN). Of the 181 patients with an initial benign microbubble/ CEUS SLN core biopsy, 7 (4%) had 2 or more axillary macrometastases found at the end of primary surgical treatment (Table 4). Thirty-one patients at Centre 2 had a true positive microbubble/ CEUS SLN core biopsy and 30 (97%) had an ALND whereas 1 (3%) had a SLNE. Of these 31 patients, 14 (45%) had 2 or more axillary LN macrometastases at the end of primary surgical treatment.

For both centres, at the end of surgical treatment the difference between the final malignant LN score of false negative versus true positive microbubble/ CEUS SLN core biopsies was statistically significant (figure 3).

Discussion

Despite the use of different ultrasound machines and variations in the methods and patient selection, the identification and core biopsy of SLN using intradermal microbubbles and CEUS in patients with breast cancer is a reproducible technique across multiple centres. Overall, the visualisation of SLN across the 4 centres ranged from 84.5% to 90.8% and a successful core biopsy from 77.5% to 88%. ~~The sensitivities of a microbubble/ CEUS core biopsy as a test to identify SLN metastases in patients with invasive breast cancer at Centres 2, 3 and 4 were within the 95% confidence intervals of Centre 1.~~ F~~The f~~actors that

appeared to adversely affect the visualisation of SLN at Centre 1 included previous surgery and disease involvement of the nipple areolar complex.

There is undoubtedly a learning curve associated with the procedure~~dure~~ and familiarity with the equipment is important as demonstrated by the data from Centre 4 where they experienced technical problems with the first 5 patients. The performance of Centre 1 radiologists also highlight~~illustrate~~ the distinct competencies of the 2 components of the procedure, namely SLN identification and SLN core biopsy. Even though identical ultrasound equipment and methods were used, the percentage of procedures with successful visualisation of SLN varied from 97% to 73%, which suggests either that not all radiologists at Centre 1 received adequate procedural training before performing the test or some found it difficult to visualise microbubbles trafficking through lymphatic channels. Six of the 7 radiologists were fairly consistent in their ability to successfully core biopsy visualised SLN, but 1 obviously struggled and only successfully retrieved lymphoid tissue in 70% of cases. Anecdotally, most radiologists accustomed to the procedure recommend that novices observe 3 cases, then perform 10 cases supervised before undertaking 30 independent procedures with an audit of their results. Once proficient, the whole procedural time is 15 to 30 minutes.

There is scope to improve the technology of CEUS. In swine models, LN metastases can be identified as areas devoid of contrast agent (11) and in a recent study of breast cancer patients, the sensitivity of CEUS as a test to identify SLN metastases using only enhancement patterns (no biopsy) was 81.8% (18). Innovations such as ultrafast ultrasound (19), super resolution imaging (20) and

improved lymphatic microbubble transit (21) may improve the ability of clinicians to visualise SLN and achieve a reliable standard.

The sensitivity~~ities~~ of a microbubble/ CEUS core biopsy as a test to identify SLN metastases in patients with invasive breast cancer and a normal grey-scale axillary ultrasound/ benign axillary LN biopsy is consistently around 50% with ~~at~~ Centres 2, 3 and 4 ~~were~~ within the 95% confidence intervals of Centre 1. ~~.~~ ~~The sensitivity of the technique as a test to identify SLN metastases is consistently around 50%,~~ As grey-scale axillary ultrasound can usefully identify approximately 50% of LN metastases (22), the addition of a microbubble/ CEUS SLN core biopsy substantially increases the overall detection rate for metastatic axillary LN. Consequently, the negative predictive value of the test is high and <5% of patients (Centres 1 and 2) with a normal grey-scale ultrasound and benign microbubble/ CEUS SLN core biopsy had 2 or more LN macrometastases detected by axillary surgery.

~~but because of the low prevalence of LN metastases in the studied patient groups, the negative predictive value of the test is high.~~

We have previously speculated that the technique has a high false negative rate~~high false negative rate~~ because the core biopsy fails to pick up small metastatic deposits~~low volume metastases~~ in SLN (13) and this may be the reason why very few patients with a false negative microbubble/ CEUS core biopsy at Centres 1 and 2 had two or more axillary LN macrometastases found at the end of primary surgical treatment. Yet, retrieving more LN tissue with a vacuum-assisted biopsy technique does not appear to appreciably increase the sensitivity of microbubbles and CEUS (23).

Alternatively, it is usual for only one SLN to be visualised and biopsied with CEUS but the median number of SLN retrieved with a surgical excision is 2 (3). In this series, 80% of surgically excised LN at Centre 1 showed evidence of a previous core biopsy and this proportion dropped to 72.6% in patients with a false negative benign microbubble/ CEUS SLN core biopsy. This raises the possibility that in the false negative cases, the second or subsequent SLN contained the metastases and perhaps more than one SLN should be actively sought with the microbubbles/ CEUS procedure.

When compared to Centre 1, a higher proportion of Centre 2 patients with a benign microbubble/ CEUS SLN core biopsy had two or more axillary macrometastases found at the end of surgical treatment (1.8 vs 4%). This may be related to the smaller patient sample size in Centre 2 or the higher prevalence of LN metastases in patients with a successful microbubble/ CEUS SLN core biopsy in Centre 2 (27% vs 22% at Centre 1). As the patient and clinicopathologic features of the tumours were similar in both centres, the greater prevalence of LN metastases at Centre 2 may be a consequence of a lower initial metastatic LN detection rate with grey-scale axillary ultrasound.

The difference in the volume of axillary disease between patients with a false negative and true positive microbubble/ CEUS SLN core biopsy at the end of primary surgical treatment was statistically significant in Centres 1 and 2. When compared to the false negative groups in Centres 1 and 2, more patients with a true positive microbubble/ CEUS SLN core biopsy had complete axillary surgery (ALND) rather than axillary conservation (SLNE). The retrieval of a greater number of LN in the true positive groups from Centres 1 and 2 may therefore have increased the total number of axillary LN metastases found at the end of

surgical treatment and influenced the final metastatic score. However, in a previous publication from Centre 1 (14~~reference blinded for review~~) comparing only patients with complete axillary surgery (ALND), the difference in the volume of axillary disease between those with a false negative and true positive microbubble/ CEUS SLN core biopsy remained stastically significant.

An argument against using CEUS to biopsy SLN in routine practice is that patients with a biopsy containing malignant cells are committed to a primary ALND for what may be a low burden of axillary disease. This can be mitigated against by offering SLNE to patients with micrometastases in the core biopsy specimen. It should also be noted that 50% of patients at Centre 1 and 45% of patients at Centre 2 with a true positive microbubble/ CEUS SLN core biopsy had 2 or more axillary macrometastases found at the end of surgical treatment and therefore using the test for patients who are not eligible for axillary conservation (7) is beneficial.

Conclusion

~~In patients with breast cancer, the identification and biopsy of SLN using intradermally injected microbubbles and CEUS is a reproducible technique~~. The results of the American College of Surgeons Oncology group Z0011 trial (6) have changed practice by showing that loco-regional control of axillary metastases is not solely dependant upon surgical excision and residual disease can be treated with adjuvant therapy. In addition, anatomic staging of breast cancer is likely to become less relevant to treatment decisions as tumour genomic and molecular assays are better understood (24). Based on recent information obtained from

Centre 1, a SLNE costs 3.6x the cost of a microbubble/ CEUS SLN core biopsy (£671.63 vs £189 respectively). Omitting axillary surgery will improve theatre utilisation (potentially allowing more cases to be added to a list) as well as reducing the anaesthetic time for each patient. In the era of axillary conservation, molecular medicine and dwindling resources ~~, which provides useful information that can influence treatment decisions. In the era of axillary conservation,~~ the combination of grey-scale axillary ultrasound and~~a~~ microbubble/ CEUS SLN core biopsy has the potential to succeed surgical staging of the axilla. Further work now needs to be undertaken to refine the procedure with protocols, standard setting and training.

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Figure Captions

Figure 1

Diagram showing the selection and flow of participants from each centre through the study. Lymph node (LN), Sentinel Lymph Node (SLN), Contrast enhanced ultrasound (CEUS), Ductal Carcinoma in situ (DCIS),

isolated tumour cells (ITC), surgical sentinel lymph node biopsy (SLNB), axillary lymph node dissection (ALND) and POSNOC – (POsitive Sentinel NOde: adjuvant therapy alone versus adjuvant therapy plus Clearance or axillary radiotherapy. A randomised controlled trial of axillary treatment in women with early stage breast cancer who have metastases in one or two sentinel nodes).

Figure 2

A. Ultrasound contrast pulse sequencing image of a SLN (white arrow) after injection of intradermal microbubbles (between 0.2 and 1 ml injected using a 26G needle with 1ml tuberculin syringe) into the UOQ periareolar area of the breast. B. Grey scale ultrasound image of the same SLN as visualised in A. Images provided by Centre 3.

Figure 3

A. Volume of axillary disease at the end of primary surgical treatment for individual patients at Centres 1 and 2 who had a false negative (FN) or true positive (TP) microbubble/ CEUS core biopsy of SLN. Axillary lymph node dissection (ALND) and sentinel lymph node excision (SLNE). B. The total volume of axillary metastases at the end of surgical treatment was determined using a scoring system (isolated tumour cells = 0.2, each LN micrometastasis = 0.5 and each LN macrometastasis = 1). The Mann-Whitney test was used to compare the volume of axillary metastases between patients with a false negative and true positive microbubble/ CEUS SLN biopsy in both centres.

Table 1

Performance statistics of 7 consultant breast radiologists at Centre 1 (Total 1349 of 1361 procedures). In 12 cases the data was incomplete and the name of the radiologist was not recorded. For radiologist no.1, one successful procedure was a fine needle aspiration biopsy rather than a core biopsy.

Table 2

Test accuracy of CEUS guided SLN biopsy using intradermally injected microbubbles in women with normal (centres 1 and 2) or indeterminate results from previous grey scale ultrasound (with or without previous biopsy). Reference standard is Sentinel Lymph node excision (SLNE) or axillary lymph node dissection (ALND). Positive predictive value (PPV) and negative predictive value (NPV) were calculated directly for centres 1 and 2 who employed consecutive recruitment, and using Bayesian methods with 22% prevalence at centres 3 and 4 as these were not a consecutively or randomly selected group. True positive (TP), false positive (FP), true negative (TN) and false negative (FN).

Table 3

Age and clinicopathological characteristics of all patients at Centre 1 (first column) with a successful microbubble/CEUS SLN core biopsy before primary surgical treatment and Centre 1 patients with false negative microbubble/ CEUS SLN core biopsies sub-divided into micrometastases

(<2mm)/ isolated tumour cells (ITC), low volume metastases and high volume metastases identified at the end of primary surgical treatment. Data are expressed as *n* (%). Estrogen receptor (ER), progesterone receptor (PR), human epidermal growth factor receptor 2 (Her2), invasive ductal carcinoma (IDC), invasive lobular carcinoma (ILC).

Table 4

Age and clinicopathological characteristics of all patients at Centre 2 (first column) with a successful microbubble/CEUS SLN core biopsy before primary surgical treatment and Centre 2 patients with false negative microbubble/ CEUS SLN core biopsies sub-divided into micrometastases (<2mm)/ isolated tumour cells (ITC), low volume metastases and high volume metastases identified at the end of primary surgical treatment. Data are expressed as *n* (%). Estrogen receptor (ER), progesterone receptor (PR), human epidermal growth factor receptor 2 (Her2), invasive ductal carcinoma (IDC), invasive lobular carcinoma (ILC).

Table 1

	Centre 1 – Consultant Breast Radiologists						
	1	**2**	**3**	**4**	**5**	**6**	**7**
Total Procedures	276	37	501	81	116	207	131
Procedures where core biopsy not attempted	0 (0%)	0 (0%)	0 (0%)	2 (2 .5%)	0 (0 %)	0 (0 %)	1 (0.8%)
Procedures with successful visualisation of SLN	269 (97.5%)	33 (89.2%)	457 (91.2%)	59 (72.8%)	94 (81%)	187 (90.3%)	118 (90.1%)
Procedures without SLN tissue retrieved	1 (0.4%)	1 (2.7%)	64 (12.8%)	2 (2.5%)	10 (8.6%)	18 (8.7%)	34 (26%)
Successful retrieval of lymphoid tissue in those with visualised SLN	268 (99.6%)	32 (97%)	393 (86%)	57 (96.6%)	84 (89.4%)	169 (90.4%)	84 (71.2%)
Total successful visualisation and	268 (97.1%)	32	393 (78.4%)	57	84	169	84

core biopsy of SLN		(86.5%)		(70.4%)	(72.4%)	(81.6%)	(64.1%)

Table 2

Centre	Prevalence of LN metastases	TP	FP	TN	FN	Sensitivity	Specificity	PPV	NPV
1	22%	84	2	635	95	46.9% (39.4-55.5%)	99.7 % (98.95-100%)	97.7% (91.9-99.7%)	87.0% (84.3-89.3%
2	27%	31	3	153	28	52.5% (39.1-65.7%)	98.1% (94.5-99.6%)	91.2% (76.3-98.1%)	84.5% (78.4-89.5%)
3	35%	13	0	52	15	46.4% (27.5-66.1%)	100% (93.2-100%)	100% (75.3-100%)	86.9% (82.4-90.3%)
4	29%	5	1	26	6	45.5% (16.7-76.6%)	96.3% (81.0-99.9%)	77.6% (31.3-96.3%)	86.2 % (78.4 -91.5%)

Table 3

		Volume of disease at the end of surgical treatment (microbubble/ CEUS false negative core biopsy)		
Centre 1	**All Patients**	**Micrometastasis/ ITC**	**Low (<2 LN macrometastases)**	**High (2 or more LN macrometastases)**
Total number of patients	816	37	46	12
		15 (41%) ALND	34 (74%) ALND	12 (100%) ALND
Median age in years (range)	61 (30-94)	61 (42-89)	55 (32-90)	53 (36-69)
Receptor Status				
ER positive	707 (87%)	32 (86%)	40 (87%)	11 (92%)
ER unknown	4 (0.4%)	0	0	0
Her-2 positive	79 (10%)	3 (8%)	2 (4%)	1 (8%)
Her-2 not recorded	13 (1.6%)	0	2 (4%)	0
ER-/PR-/Her-2-	72 (10%)	4 (10.8%)	4 (9%)	0
ER-/PR-/HER-2+	28 (3%)	1 (2.7%)	1 (2%)	1 (8%)
Invasive Tumour Size				
DCIS + microinvasion	5 (0.6%)	0	0	0
T1	472 (58%)	19 (51%)	17 (37%)	4 (33%)
T2	199 (24%)	10 (27%)	18 (39%)	5 (42%)
T3	29 (4%)	5 (14%)	3 (7%)	1 (8%)
Multifocal	111 (14%)	3 (8%)	8 (17%)	2 (17%)
Unknown	0	0	0	0
Tumour Grade				
Grade 1	180 (22%)	6 (16%)	7 (15%)	4 (33%)
Grade 2	392 (48%)	20 (54%)	21 (46%)	6 (50%)
Grade 3	209 (26%)	11 (30%)	14 (30%)	1 (8%)
Mixed grade	29 (4%)	0	4 (9%)	1 (8%)

Unknown	1 (0.1%)	0	0	0
Tumour Type				
IDC	660 (81%)	23 (62%)	38 (83%)	11 (92%)
ILC	103 (13%)	11 (30%)	6 (13%)	1 (8%)
Other	29 (4%)	1 (3%)	1 (2%)	0
Mixed	17 (2%)	2 (5%)	1 (2%)	0
Unknown	2 (0.2%)	0	0	0

Table 4

		Volume of disease at the end of surgical treatment (microbubble/ CEUS false negative core biopsy)		
Centre 2	**All Patients**	**Micrometastasis/ ITC**	**Low (<2 LN macrometastases)**	**High (2 or more LN macrometastases)**
Total number of patients	215	10	11	7
		2 (20%) ALND	10 (91%) ALND	7 (100%) ALND
Median age in years (range)	64 (31-93)	63.5 (37-93)	62 (38-91)	55 (47-72)
Receptor Status				
ER positive	176 (82%)	10 (100%)	8 (73%)	6 (86%)
ER unknown	6 (3%)	0	1 (9%)	1 (14%)
Her-2 positive	29 (13%)	1 (10%)	0	1 (14%)
Her-2 not recorded	8 (4%)	0	1 (9%)	1 (14%)
ER-/PR-/Her-2-	23 (11%)	0	1 (9%)	0
ER-/PR-/HER-2+	10 (5%)	0	0	0
Invasive Tumour Size				
DCIS + microinvasion	0	0	0	0
T1	118 (55%)	2 (20%)	6 (55%)	2 (29%)
T2	71 (33%)	6 (60%)	4 (36%)	5 (71%)
T3	9 (4%)	0	0	0
Multifocal	17 (8%)	2 (20%)	0	0
Unknown	0	0	1 (9%)	0
Tumour Grade				
Grade 1	35(16%)	1 (10%)	0	2 (29%)
Grade 2	102 (47%)	6 (60%)	5 (45.5%)	1 (14%)
Grade 3	68 (32%)	2 (20%)	4 (36%)	4 (57%)

Mixed grade	4 (2%)	0	0	0
Unknown	6 (3%)	0	2 (18%)	0
Tumour Type				
IDC	160 (74%)	7 (70%)	8 (73%)	5 (71%)
ILC	24 (11%)	2 (20%)	1 (9%)	0
Other	16 (7%)	0	0	0
Mixed	13 (6%)	1 (10%)	1 (9%)	2 (29%)
Unknown	2 (1%)	0	1 (9%)	0

Figure 1

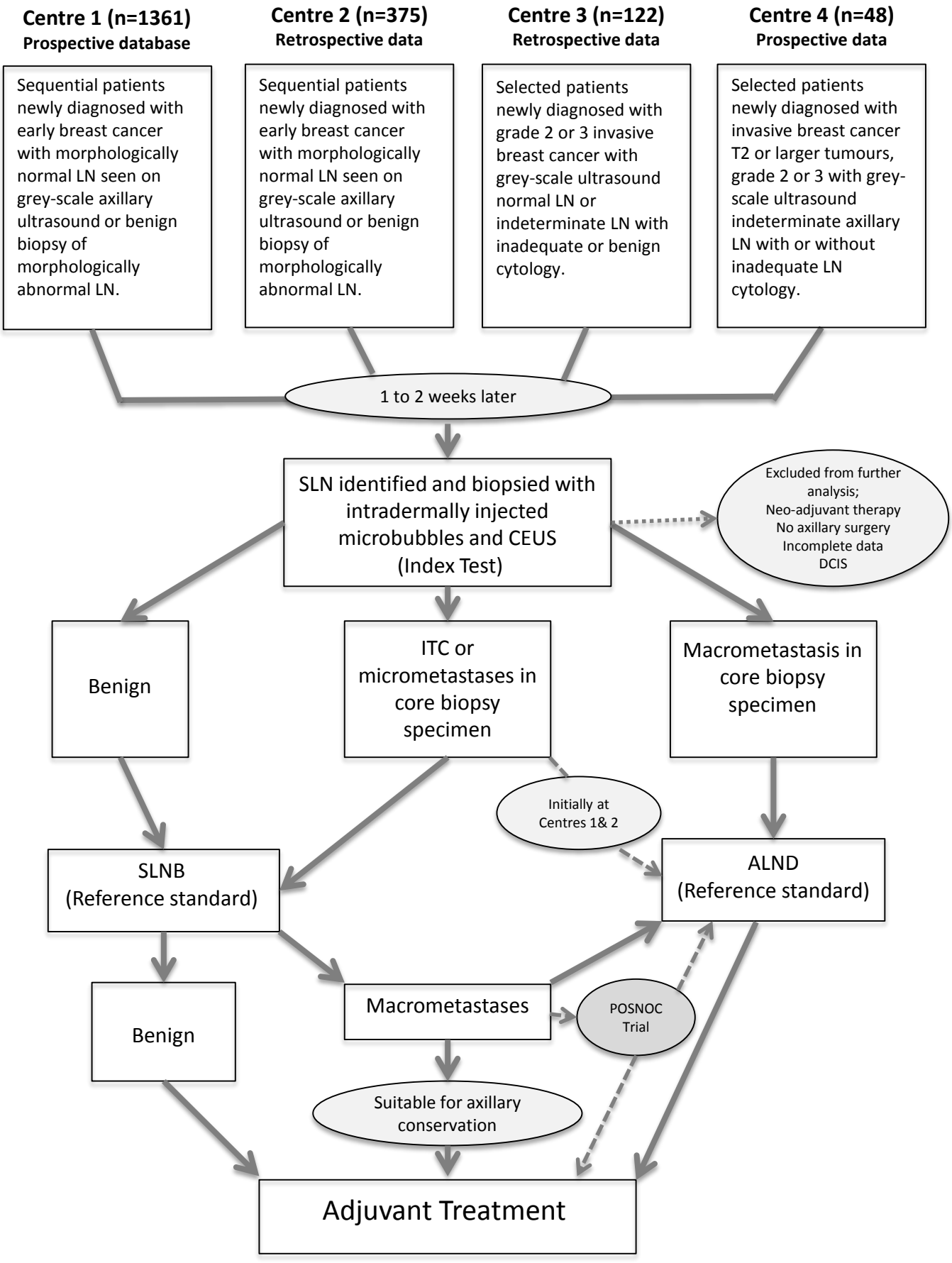
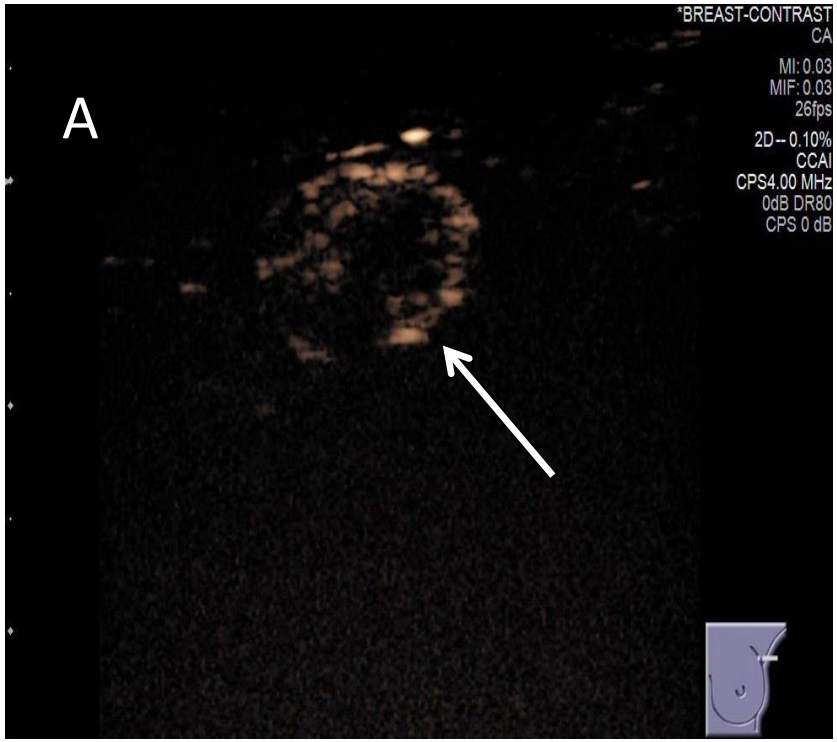

Centre 1 (n=1361)
Prospective database
Sequential patients newly diagnosed with early breast cancer with morphologically normal LN seen on grey-scale axillary ultrasound or benign biopsy of morphologically abnormal LN.
Centre 2 (n=375)
Retrospective data
Sequential patients newly diagnosed with early breast cancer with morphologically normal LN seen on grey-scale axillary ultrasound or benign biopsy of morphologically abnormal LN.
Centre 3 (n=122)
Retrospective data
Selected patients newly diagnosed with grade 2 or 3 invasive breast cancer with grey-scale ultrasound normal LN or indeterminate LN with inadequate or benign cytology.
Centre 4 (n=48)
Prospective data
Selected patients newly diagnosed with invasive breast cancer T2 or larger tumours, grade 2 or 3 with grey-scale ultrasound indeterminate axillary LN with or without inadequate LN cytology.
1 to 2 weeks later
SLN identified and biopsied with intradermally injected microbubbles and CEUS (Index Test)
Excluded from further analysis;
Neo-adjuvant therapy
No axillary surgery
Incomplete data
DCIS
Benign
ITC or micrometastases in core biopsy specimen
Macrometastasis in core biopsy specimen
Initially at Centres 1& 2
SLNB (Reference standard)
ALND (Reference standard)
Benign
Macrometastases
POSNOC Trial
Suitable for axillary conservation
Adjuvant Treatment

Figure 2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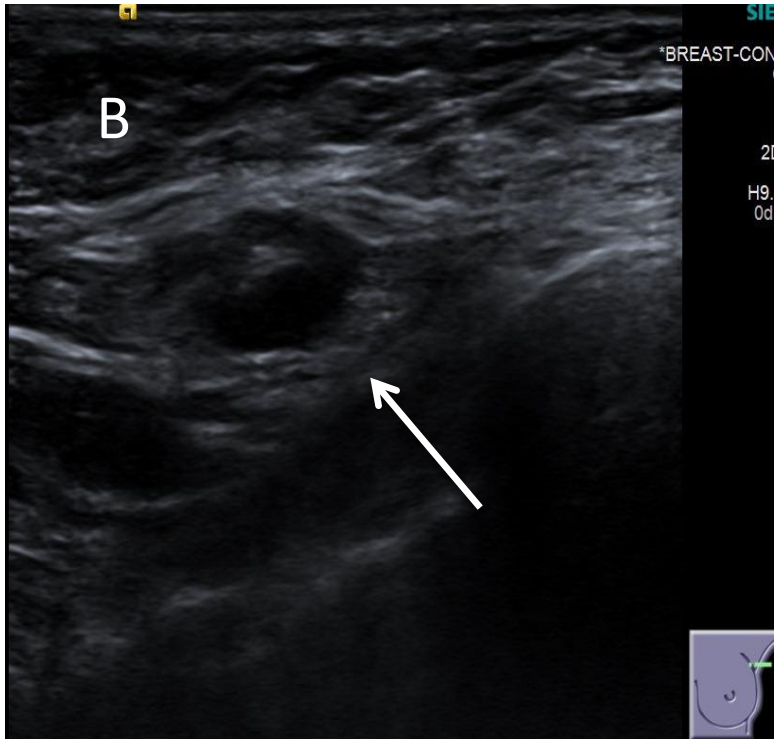
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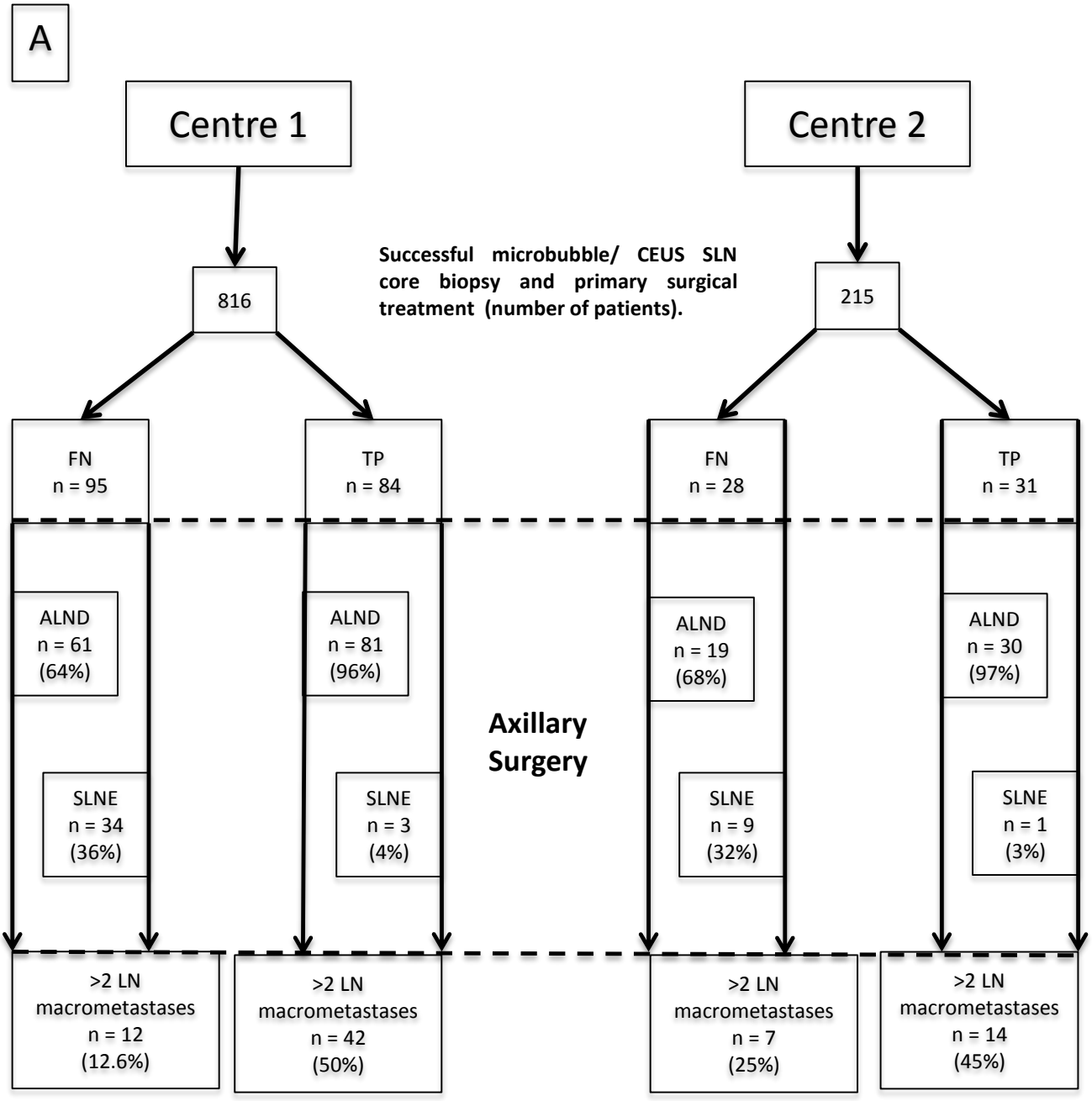
Figure 3



B

Centre	no. of true positives	Median LN score (IQR)	no. of false negatives	Median LN score (IQR)	P-value
1	84	1.75 (1 – 3)	95	1 (0.5 – 1)	<0.0005
2	31	1.5 (1 – 2)	28	1 (0.5 – 2)	0.01